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Simultaneous determination of the enantiomers of pimobendan and its main metabolite in rat plasma by high-performance liquid chromatography

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ABSTRACT

A high-performance liquid chromatographic method for the simultaneous analysis of the enantiomers of pimobendan and its main metabolite, with an *n*-hexane–ethanol–acetic acid solvent system, has been developed. After solid-phase extraction from plasma, the enantiomers were separated from each other using a Sumichiral OA-4400 column, which is commercially available and contains a chiral stationary phase composed of (*S*)-proline and (*S*)-1-(α-naphthyl)ethylamine coated on silica. The enantiomers were detected with a fluorescence detector (excitation at 330 nm, emission at 415 nm). The intra- and inter-day precision studies showed good reproducibilities: the coefficients of variation were less than 10.3% for pimobendan enantiomers and 13.0% for metabolite enantiomers. The calibration curves were linear ($r^2 > 0.996$) in the concentration range 1.25–200 ng/ml. The minimum measurable level was 125 pg per 100 μl of plasma. The method was used in a preliminary pharmacokinetic study in three male rats after intravenous administration of racemic pimobendan (2 mg/kg).

INTRODUCTION

Pimobendan, 4,5-dihydro-6-[2-(*p*-methoxyphenyl)-5-benzimidazolyl]-5-methyl-3(2*H*)-pyridazinone (Fig. 1), synthesized by Dr. Karl Thomae (Biberach, Germany), is a new positive inotropic agent with vasodilatory properties [1,2]. It is also known that a main metabolite (Fig. 1, metabolite I) possesses the same effects as the parent compound [3]. Recently, it has been reported that many chiral drugs show enantioselective pharmacokinetics and/or pharmacodynamics, and so enantioselective analytical methods are required [4–6]. Pimobedan has an asymmetric carbon atom in the pyridazinone ring (see Fig. 1) and exhibits enantioselective pharmacological effects *in vitro* and *in vivo* [7,8].

This paper describes the development of an HPLC method for the enantioselective determination of pimobendan and metabolite I to elucidate the pharmacokinetics of the enantiomers.

EXPERIMENTAL

Reagents

Racemates of pimobendan and metabolite I and enantiomers of pimobendan were supplied by Dr. Karl Thomae. The purities of the enantiomers were higher than 99.7%. Bond Elut (PH, 1 cc, Analytichem International, Harbor City, CA, USA) was obtained from GL Sciences (Tokyo, Japan). HPLC-grade *n*-hexane and ethanol were obtained from Wako (Osaka, Japan).

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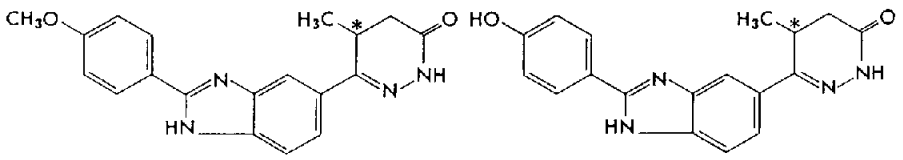

(A) (B)

Fig. 1. Structure of pimobendan (A) and metabolite I (B). Chiral centres are indicated by asterisks.

All other chemicals were of analytical grade, and Milli-Q water was used. All reagents were used without further purification.

Stock solutions

Stock solutions of 4000, 2000, 1000, 250, 50 and 25.0 ng/ml were prepared by dissolving racemic pimobendan, (+)-pimobendan, (−)-pimobendan and racemic metabolite I in ethanol. For direct HPLC injection of each substance, the stock solution was evaporated under a gentle nitrogen stream at 60°C and reconstituted with ethanol–*n*-hexane (1:1, v/v).

Extraction procedure

Bond Elut, set to an Adsorbex sample processing unit (E. Merck, Darmstadt, Germany), was pre-treated by washing with 3 ml of methanol and 3 ml of water. To 100 μl of plasma sample or calibration standard, 1 ml of phosphate buffer (0.1 *M*, pH 9.5) was added. The mixture was loaded onto pre-treated Bond Elut under low vacuum. After washing with 3 ml of water, it was dried for at least 15 min by air under high vacuum. The substances were eluted with 1 ml of methanol under low vacuum. The eluate was evaporated under a gentle nitrogen stream at 60°C, and the residue was reconstituted with 200 μl of ethanol–*n*-hexane (1:1, v/v). A 100-μl volume of the solution was injected to the chromatograph.

HPLC apparatus

An LC-6A HPLC system (Shimadzu, Kyoto, Japan), which consisted of two constant-flow pumps (LC-6A), an automatic sample injector (SIL-6B), a column oven (CTO-6A), a fluorescence detector (RF-550), a system controller (SCL-6B) and a reporting integrator (C-R4A), was used. A Sumichiral OA-4400 analytical column (250 mm × 4.6 mm I.D., 5 μm particle size; Sumika Chemical Analysis Service, Osaka, Japan) was used for separation of the enantiomers. The mobile phase was *n*-hexane–ethanol–acetic acid (300:120:1, v/v/v), and the flow-rate was 0.8 ml/min. The column temperature was maintained at 40°C. The excitation and emission wavelengths of the detector were set at 330 and 415 nm, respectively. To increase the fluorescence intensity of pimobendan and metabolite I, ethanol acetic acid (1:1, v/v) was added to the column eluent through a T-connector at a flow-rate of 0.3 ml/min.

Enantiometric elution order

The elution order of the enantiomers was determined by direct injection of each substance and chiral detection (Shodex OR-1, Showa denko, Tokyo, Japan). However, we could not obtain enantiomers of metabolite I, so that their elution order was determined only by optical rotation.

Recovery from plasma

The recoveries of the enantiomers of pimobendan and metabolite I from plasma were examined. Spiked samples were prepared by adding known amounts of racemic pimobendan and racemic metabolite I to blank plasma. The concentrations of the samples were 400, 100 and 5.0

ng/ml for racemic pimobendan and racemic metabolite I. The samples were analysed as described. The peak heights obtained were compared with those obtained by direct injection of each substance without extraction.

Calibration curve

To make calibration standards, 0.1 ml of each stock solution was added to 0.9 ml of rat blank plasma. The concentrations of the calibration standards were 400, 200, 100, 25.0, 5.0 and 2.5 ng/ml for racemic pimobendan and racemic metabolite I. Calibration standards were analysed, and the calibration curves were constructed by plotting the peak heights against the known concentrations, using least-squares regression.

Intra-day and inter-day precision studies

Spiked samples were prepared at concentrations of 400, 200, 100, 25.0, 5.0 and 2.5 ng/ml for racemic pimobendan and racemic metabolite I. The spiked samples were stored at −20°C until analysis. The reproducibility and accuracy for each enantiomer were determined by sample analysis. The inter-day study was performed for two weeks.

Application of the method

Plasma levels of the enantiomers of pimobendan and metabolite I were determined in rats after intravenous administration of racemic pimobendan (2 mg/kg). Three Sprague–Dawley male rats (218–240 g; Japan SLC, Shizuoka, Japan) were fasted overnight and used for the study. The administration solution (1 mg/ml) was prepared by dissolving the substance in 5% mannitol saline solution acidified with methane sulphonate. Blood (*ca.* 0.4 ml) was collected from the retro-orbital venous plexus at 5, 15 and 30 min and 1, 2 and 4 h after administration, under halothane anaesthesia. After centrifugation, plasma was obtained and stored at −20°C until analysis. For pharmacokinetic data analysis, C_{max} (plasma level 5 min after administration), AUC (area under the plasma level–time curve) and MRT (mean residence time) were estimated. AUC was calculated by the trapezoidal rule using 0–4 h data. MRT was calculated by the area under the first moment of the plasma level–time curve divided by AUC. C_{max} values were determined from observed values. The statistical significance was evaluated using Student's unpaired *t*-test.

RESULTS AND DISCUSSION

In this study, our aim was to develop an enantioselective determination method for pimobendan and its O-demethyl metabolite, metabolite I. We focused in particular on simultaneous determination, because the pharmacological effects observed during pimobendan treatment were considered to be partially due to metabolite I. The enantiomers could be separated from each other using a commercially available chiral column without complicated extraction procedures. Typical chromatograms of rat blank plasma spiked with each enantiomer, and of plasma from a rat 1 h after administration of racemic pimobendan are shown in Fig. 2. No significant interference peaks were observed. The retention times of (−)- and (+)-pimobendan as well as (−)- and (+)-metabolite I, which were determined by direct injection of each substance and optical rotation, were 14.4, 15.1 and 27.8 and 29.6 min, respectively. The calculated resolution factors were 0.79 for pimobedan enantiomers and 0.89 for metabolite I enantiomers; however, there was no problem in the quantitative determination.

The extraction recoveries using Bond Elut PH were higher than 94.0% for (−)-pimobendan and 94.4% for the (+)-enantiomer, 82.5% for (−)-metabolite I and 84.8% for the (+)-enantiomer. The coefficient of variation (C.V.) for the mean peak height was less than 8.1% for pimobendan enantiomers and 8.7% for metabolite I enantiomers, demonstrating good reproducibilities (Table I).

Intra-day (n = 4) and inter-day (n = 5) reproducibility and accuracy were checked for concentrations of 200, 100, 50, 12.5, 2.5 and 1.25 ng/ml for each enantiomer. The C.V. of the determined values were less than 10.29% for (−)- pimobendan and 8.76% for the (+)-enantiomer, 8.63% for (−)-metabolite I and 13.04% for the (+)-

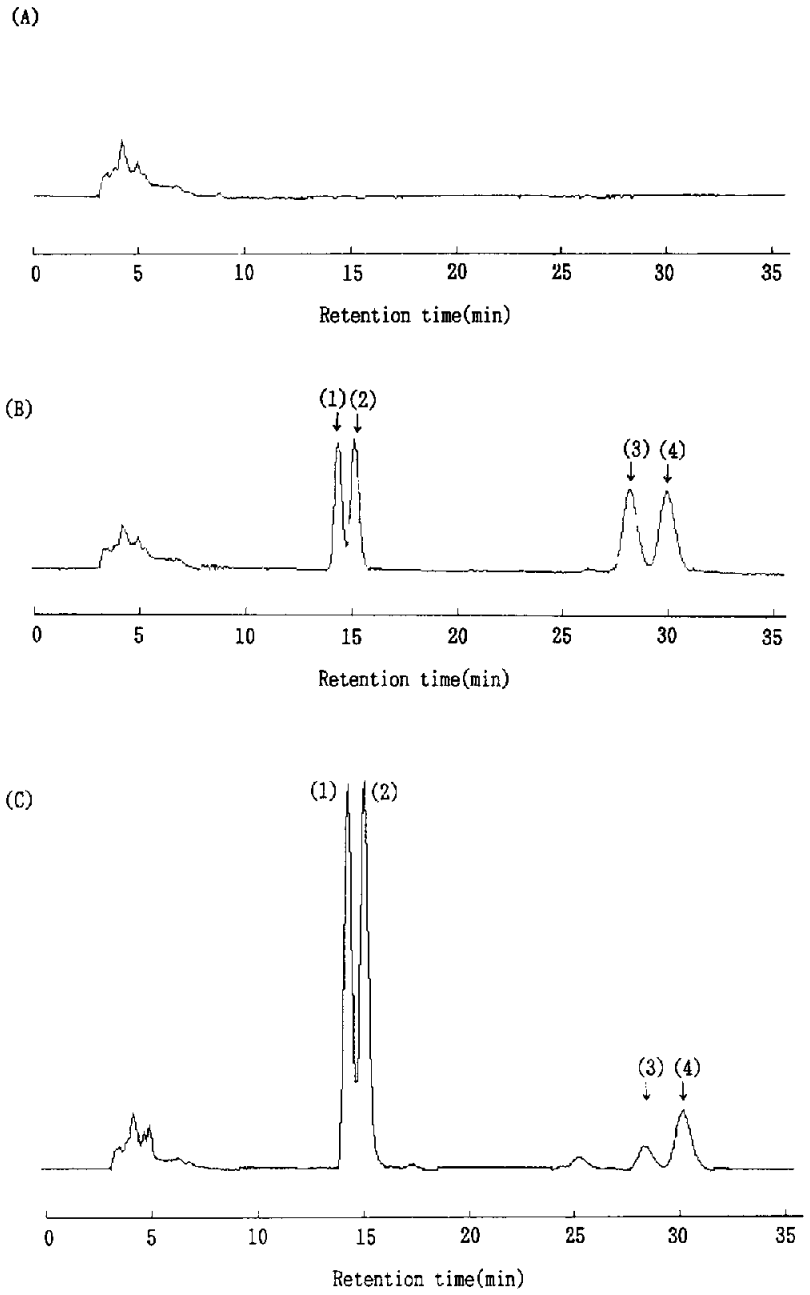

Fig. 2. Typical chromatograms of drug-free plasma (A), plasma spiked with 12.5 ng/ml of each enantiomer (B), and plasma from a rat 1 h after intravenous administration of racemic pimobendan (2 mg/kg) (C) after extraction. Peaks: 1 = (−)-pimobendan; 2 = (+)-pimobendan; 3 = (−)-metabolite I; 4 = (+)-metabolite I.

enantiomer. In addition, the accuracy was estimated to be 94.40–111.20% (Table II). Calibration graphs for each enantiomer showed good linearity in the concentration range 1.25–200 ng/ml plasma. The correlation coefficient (r^2) was more than 0.9985 for (−)-pimobendan, 0.9983 for the (+)-enantiomer, 0.9972 for (−)-metabolite I and 0.9964 for the (+)-enantiomer. These

TABLE I

EXTRACTION RECOVERY STUDY

Results are mean ± S.D. of peak height (n = 4).

Compound	Concentration (ng/ml)	Non-extract	C.V. (%)	Extract	C.V. (%)	Recovery (%)
(−)-Pimobendan	200.0	139 454.0 ± 11 348.2	8.1	131 150.8 ± 3315.2	2.5	94.6 ± 9.3
	50.0	34 850.0 ± 1936.8	5.6	32 634.3 ± 1063.7	3.3	94.0 ± 7.8
	2.5	1523.0 ± 112.1	7.4	1656.8 ± 96.9	5.8	109.5 ± 13.2
(+)-Pimobendan	200.0	149 082.8 ± 10 391.1	7.0	140 363.3 ± 3132.3	2.2	94.6 ± 8.6
	50.0	37 114.3 ± 1740.5	4.7	34 952.5 ± 1192.7	3.4	94.4 ± 7.0
	2.5	1662.5 ± 121.8	7.3	1746.3 ± 104.1	6.0	105.8 ± 13.3
(−)-Metabolite I	200.0	70 560.0 ± 4493.0	6.4	58 170.5 ± 949.7	1.6	82.8 ± 6.6
	50.0	17 312.5 ± 782.9	4.5	14 248.0 ± 352.3	2.5	82.5 ± 5.6
	2.5	672.8 ± 46.4	6.9	612.3 ± 53.4	8.7	91.8 ± 14.6
(+)-Metabolite I	200.0	73 104.3 ± 4585.1	6.3	61 899.5 ± 971.5	1.6	85.0 ± 6.6
	50.0	17 986.3 ± 863.1	4.8	15 208.0 ± 346.3	2.3	84.8 ± 5.6
	2.5	715.8 ± 44.8	6.3	666.3 ± 52.8	7.9	93.7 ± 13.5

results showed good reproducibility and accuracy using the present analysis method. The limit of detection of each enantiomer in plasma was 1.25 ng/ml, representing 62.5 pg injected.

We used this method to measure plasma levels of the enantiomers of pimobendan and metabolite I in rats. After intravenous administration of racemic pimobendan (2 mg/kg), the plasma level profiles of (+)-pimobendan and the (−)-form showed no difference, that is, there were no significant differences in the C_{max}, AUC or MRT values. On the other hand, the AUC and C_{max} values of (+)-metabolite I were significantly higher than those of the (−)-form (Table III). The AUC ratio (metabolite I enantiomer/pimobendan enantiomer) was significantly higher for the (+)-forms than the (−)-forms ($p < 0.05$, Fig. 3): the value for the (−)-forms was 0.0823 ± 0.0064, and that for the (+)-forms was 0.2260 ± 0.0450 (mean ± S.D., n = 3 in both cases).

These results suggest enantioselective pharmacokinetics of metabolite I, and additional studies are needed to clarify the mechanism of the enantioselectivity.

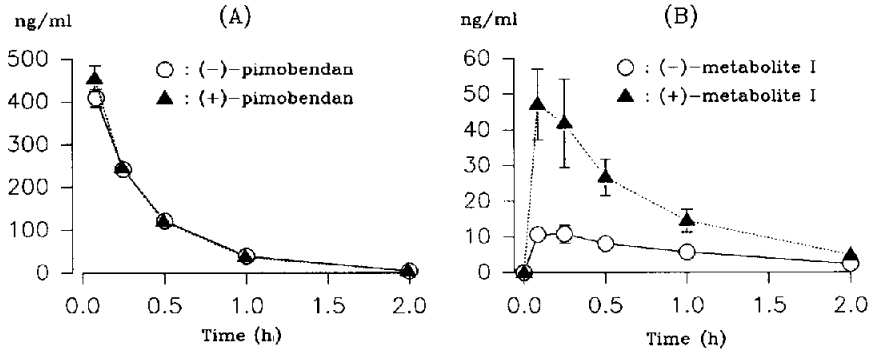

Fig. 3. Plasma levels of the enantiomers of pimobendan (A) and metabolite I (B) in rats after intravenous administration of racemic pimobendan (2 mg/kg). Each point represents mean ± S.D. (n = 3).

TABLE II

INTRA-DAY AND INTER-DAY REPRODUCIBILITIES FOR PIMOBENDAN AND METABOLITE I ENANTIOMERS

Compound	Spiked concentration (ng/ml)	Intra-day (n = 4)			Inter-day (n = 5)		
		Determined concentration (mean ± S.D.) (ng/ml)	C.V. (%)	Accuracy[a] (%)	Determined concentration (mean ± S.D.) (ng/ml)	C.V. (%)	Accuracy[a] (%)
(−)-Pimobendan	200.00	198.48 ± 10.01	5.04	99.24	199.45 ± 5.45	2.73	99.73
	100.00	102.54 ± 5.68	5.54	102.54	101.96 ± 1.31	1.28	101.96
	50.00	51.54 ± 1.22	2.37	103.08	49.07 ± 3.67	7.48	98.14
	12.50	12.67 ± 0.57	4.50	101.36	12.31 ± 0.83	6.74	98.48
	2.50	2.50 ± 0.21	8.40	100.00	2.54 ± 0.05	1.97	101.60
	1.25	1.29 ± 0.06	4.65	103.20	1.36 ± 0.14	10.29	108.80
(+)-Pimobendan	200.00	197.71 ± 8.40	4.25	98.86	200.61 ± 3.56	1.77	100.31
	100.00	102.20 ± 5.33	5.22	102.20	102.57 ± 2.16	2.11	102.57
	50.00	51.50 ± 1.29	2.50	103.00	48.84 ± 3.55	7.27	97.68
	12.50	12.73 ± 0.43	3.38	101.84	12.43 ± 0.89	7.16	99.44
	2.50	2.54 ± 0.19	7.48	101.60	2.51 ± 0.04	1.59	100.40
	1.25	1.34 ± 0.10	7.46	107.20	1.37 ± 0.12	8.76	109.60
(−)-Metabolite I	200.00	199.46 ± 6.32	3.17	99.73	196.16 ± 10.58	5.39	98.08
	100.00	103.39 ± 1.67	1.62	103.39	102.06 ± 5.30	5.19	102.06
	50.00	50.66 ± 1.42	2.80	101.32	48.69 ± 3.65	7.50	97.38
	12.50	12.69 ± 0.17	1.34	101.52	12.36 ± 0.65	5.26	98.88
	2.50	2.36 ± 0.14	5.93	94.40	2.52 ± 0.11	4.37	100.80
	1.25	1.19 ± 0.08	6.72	95.20	1.39 ± 0.12	8.63	111.20
(+)-Metabolite I	200.00	199.04 ± 6.59	3.31	99.52	197.42 ± 10.34	5.24	98.71
	100.00	103.12 ± 1.77	1.72	103.12	100.07 ± 3.23	3.23	100.07
	50.00	51.16 ± 1.66	3.24	102.32	48.54 ± 4.10	8.45	97.08
	12.50	12.72 ± 0.24	1.89	101.76	12.23 ± 0.71	5.81	97.84
	2.50	2.41 ± 0.12	4.98	96.40	2.48 ± 0.09	3.63	99.20
	1.25	1.28 ± 0.08	6.25	102.40	1.38 ± 0.18	13.04	110.40

[a] Accuracy = (determined concentration/spiked concentration) × 100%.

TABLE III

PHARMACOKINETIC PARAMETERS

Each value represents mean ± S.D. (n = 3).

Compound	C_{max}[a] (ng/ml)	AUC (0–4 h) (ng · h/ml)	MRT (0–4 h) (h)
(−)-Pimobendan	408.9 ± 20.8	175.0 ± 15.6	0.46 ± 0.02
(+)-Pimobendan	454.2 ± 30.3	188.3 ± 5.4	0.44 ± 0.01
(−)-Metabolite I	7.8 ± 3.9	14.3 ± 0.3	0.94 ± 0.14
(+)-Metabolite I	47.1 ± 9.9[b]	42.5 ± 7.4[c]	0.78 ± 0.07

[a] Plasma level 5 min after administration.
[b] $p < 0.01$.
[c] $p < 0.05$.

CONCLUSION

The analytical method described is a convenient HPLC method for the simultaneous determination of the enantiomers of pimobendan and its main metabolite. Because it is simple and shows good reproducibility, this method is applicable to pharmacokinetic studies on rats.

REFERENCES

1 J. C. A. van Meel and W. Diederen, *J. Cardiovasc. Pharmacol.*, 339 (1989) 575.
2 K. Fujino, N. Sperelakis and R. J. Solaro, *Circ. Res.*, 63 (1988) 911.

3 D. Brunkhorst, H. von der Leyen, W. Meyer, R. Nigbur, C. Schmidt-Schumacher and H. Scholz, *Naunyn-Schmiedeberg's Arch. Pharmacol.*, 339 (1989) 575.
4 E. J. Ariëns, *Eur. J. Clin. Pharmacol.*, 26 (1984) 663.
5 D. E. Drayer, *Clin. Pharmacol. Ther.*, 40 (1986) 125.
6 F. Jamali, R. Mehvar and F. M. Pasutto, *J. Pharm. Sci.*, 78 (1989) 695.
7 K. Kanai, T. Hayashi, K. Ogino and H. Kohei, *Jpn. J. Pharmacol.*, 58 (Suppl. I) (1992) 270.
8 K. Fujino, N. Sperelakis and R. J. Solaro, *J. Pharmacol. Exp. Ther.*, 247 (1988) 519.